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Kallikreins, steroid hormones and ovarian cancer: is there a link?

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Kallikreins are a subgroup of serine proteases with diverse physiological functions. The human kallikrein gene family has now been fully characterized and includes 15 members tandemly located on chromosome 19q13.4. Strong experimental evidence supports a link between kallikreins and endocrine malignancies and especially, ovarian cancer. Three new kallikreins have been shown to be potential diagnostic and prognostic markers for ovarian cancer. Many other kallikreins are also differentially expressed in ovarian cancer, and preliminary reports underline their possible prognostic value. The mechanism by which kallikreins could be involved in ovarian cancer pathology is not known. A likely link could be their regulation through the steroid hormone receptor pathway. Most kallikreins are under sex steroid hormonal regulation in cancer cell lines. Given the co-expression of many kallikreins in ovarian cancer, it is reasonable to postulate that kallikreins are involved in a cascade enzymatic pathway that plays a role in cancer progression. A multiparametric kallikrein expression profile may be a useful tool for ovarian cancer diagnosis/prognosis when used either alone or in conjunction with existing markers.

Key words: **Kallikreins, metabolism - Tumor markers, biological - Ovarian neoplasms, physiopathology - Prostatic neoplasms, physiopathology - Breast neoplasms, physiopathology - Steroids - Prognosis.**

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Ovarian cancer

Ovarian cancer represents a great clinical challenge in gynecological oncology. Since most patients are asymptomatic until the disease has metastasized, 2/3 are diagnosed in advanced stages, with survival rates of less than 20%.[1, 2] In the United States, around 23,000 new cases of ovarian cancer and about 14,000 deaths from the disease are diagnosed annually, giving it the highest mortality rate of all gynecological malignancies.

Currently, the only tumor marker that has a well-defined and validated role in the management of ovarian cancer is CA125, a large glycoprotein of unknown function.[3] Serum CA125 has been evaluated in the screening for ovarian cancer, differentiation between benign and malignant ovarian masses and prognosis.[4-6] However, it does not yet have a clear place in diagnosis, prognosis, or making treatment decisions.[3] A recent study suggested that CA125 could be used for prediction of optimal primary tumor cytoreduction, but only in stage III tumors.[7] In addition to ovarian cancer, high levels of CA125

TABLE I.—*The official gene and protein names and hormonal regulation pattern of members of the human kallikrein gene family.*

Official gene name	Official protein name	Upregulation hormone	Cell line model	Reference
KLK1	hK1	Uncertain		23-25
KLK2	hK2	Androgen, progestin	LNCap BT-474 T-47D	26
KLK3	hK3	Androgen, progestin	LNCaP BT-474 T-47D	27-33
KLK4	hK4	Androgen, progestin (prostate) Estrogen, progestin (uterine cancer)	LNCap BT-474 KLE	34-38
KLK5	hK5	Estrogen, progestin > androgen	BT-474	39
KLK6	hK6	Estrogen, progestin > androgen	BT-474	40-43
KLK7	hK7	Estrogen, glucocorticoid	BT-474	44, 45
KLK8	hK8	Estrogen > glucocorticoid	BT-474	46, 47
KLK9	hK9	Estrogen, progesterone	BT-474 T-47D MCF-7 BG-1	48, 49
KLK10	hK10	Estrogen, progestin > androgen	BT-474	50-52
KLK11	hK11	Estrogen > androgen > progestin	BT-474	53-55
KLK12	hK12	Estrogen, glucocorticoid	BT-474 LNCaP T-47D	56
KLK13	hK13	Androgen > progestin > estrogen but in BT-474 Estrogen > androgen > progestin	BT-474	57
KLK14	hK14	Androgen, progestin > estrogen		58
KLK15	hK15	Androgen > progestin, estrogen	LNCap	59

were found in 1% of the normal population, 6% of patients with benign disease and 28% of patients with non-gynecological malignancies.[8]

Many potential new serum markers for ovarian cancer have been evaluated, either alone or in combination with CA125, including inhibin, prostasin, OVX1, LASA, CA15.3 and CA72-4.[9-13] These new markers do not have a well-defined contribution at present, and only the combination of CA125 with ultrasonography yields the highest available sensitivity and specificity at present.[7] Consequently, there is a need to develop new biomarkers which can assist in prognosis, in reaching treatment decisions, in monitoring response after treatment and in identifying relapse during routine follow-up.

It is now widely accepted that no single biomarker will provide all the necessary information for diagnosis, prognosis and development of treatment strategies in patients with ovarian and other cancers. Instead, research is now focussing on developing multiparametric cancer panels.

Although newly identified markers for ovarian cancer might not be sufficient alone, the development of a panel of markers that can be used together in a multiparametric strategies, in combination with bioinformatics, might be one solution.[14, 15] Jacobs *et al.*[16] recently reported the first study with annual multimodal screening for 3 years.

The human kallikrein gene family

The term "kallikrein" was introduced in the 1930's to describe proteolytic enzymes which can release small vasoactive peptides from high molecular weight precursors. There are 2 categories of kallikrein enzymes. Plasma kallikrein is encoded by a single gene on chromosome 4. This enzyme (a serine protease), releases the vasoactive peptide bra-

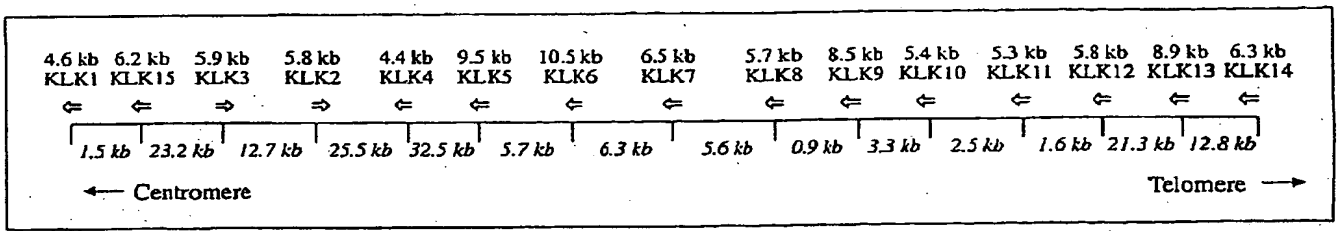

Fig. 1.—Schematic representation of the human kallikrein gene locus on chromosome 19q13.4. The direction of transcription of kallikreins are indicated by the arrows. Gene lengths (in approximate kilobases) are indicated above each gene, and the distance between the genes are indicated below. Official gene names were used for all genes. Figure is not drawn to scale.

dykinin from a high molecular weight precursor synthesized in the liver.[17] The human tissue kallikreins are a family of genes localized on chromosome 19, which also encode for serine protease enzymes. One of these enzymes (pancreatic/renal kallikrein) releases lysyl-bradykinin (kallidin) from a low molecular weight protein precursor. In this review, we will focus only on the human tissue kallikrein gene family; plasma kallikrein will not be discussed further.

Based on the original definition of kallikreins, which is based on the kininogenase activity of these enzymes, only pancreatic/renal kallikrein fulfills this criterion. Until a few years ago, 2 other enzymes, human glandular kallikrein 2 and human kallikrein 3 (PSA), were also classified as members of the human tissue kallikrein gene family, based on a number of significant homologies and similarities with pancreatic/renal kallikrein. More recently, other genes, encoding for similar enzymes (see below), are also classified as members of the human kallikrein gene family. This classification is not based on the functional definition of kallikreins but rather, on structural criteria and map location. Based on the newer definition, the number of genes that are included in the human tissue kallikrein gene family, originally thought to be much smaller than similar families found in rodents, is new increased to 15, a number that is comparable to homologous families found in rat and mouse.[18-22] A list of the official names of all kallikrein genes and proteins is included in Table I, and a schematic diagram showing the human tissue kallikrein gene locus on chromosome 19q13.4 is shown in Figure 1.

All known kallikrein genes map within an approximately 300 Kb region and the lengths of the genes, the distances between them as well as the direction of transcription have now been accurately defined.[48, 60] Telomeric from the last kallikrein gene identified (KLK14), we cloned a gene that belongs to the Siglec multigene family.[58] This finding suggests that this genomic region defines the end of the kallikrein gene family and the beginning of another family (the Siglec family of genes).[61] Centromeric from the KLK1 gene is located another novel gene named "testicular acid phosphatase" (ACPT) which is not a serine protease and appears to indicate the end of the kallikrein gene family from this end.[62]

The genomic organization of each one of these kallikrein genes is very similar and is described in details elsewhere.[22, 63] In short, all genes encode for putative secreted serine proteases and have 5 coding exons of similar lengths. All genes share significant sequence homologies at both the DNA and amino acid level and many of them are regulated by steroid hormones (see below). Despite these similarities, the tissue expression of these genes is quite different. Some genes are expressed in very few tissues, while others are abundantly expressed in most tissues. Detailed tissue expression data can be found in recent reviews.[22, 63]

In order to simplify communication, an international group of scientists working in the field has established uniform nomenclature for the kallikrein genes and their encoded proteins.[64] In this manuscript, the official nomenclature for these genes and proteins is used throughout.

Promoter and enhancer regions of kallikreins

Steroid hormones, acting through their receptors, play important roles in normal development and function of many organs. In addition, they seem to be involved in the pathogenesis of many types of cancer. As discussed below, many kallikreins are differentially regulated in cancer and their expression is controlled by steroid hormones. Extensive analysis of kallikrein gene promoters and characterization of hormone response elements is the first step in our understanding of how kallikreins might be involved in the pathogenesis of hormonally-dependent cancers. Also, as is the case with KLK3 (PSA) and KLK2, the use of tissue-specific promoters to drive therapeutic gene expression in target cells is a novel approach for gene therapy.[65] Besides KLK3 and KLK2, no other kallikrein gene promoter has been functionally tested.

Foot printing and mutation analyses have confirmed the presence of a TATA-box and a GC-box at the early promoter region of the prostate-specific antigen (KLK3) gene.[27, 66] Transfection experiments with deletion constructs revealed the presence of 2 androgen response elements (ARE-I and ARE-II) at positions -170 and -400 of the KLK3 promoter.[28] Another ARE (ARE-III) was mapped in the far upstream enhancer region of the gene and shown to be functional and tissue-specific.[29, 30] More recently, 5 additional low affinity AREs have been identified close to ARE-III,[67] and 3 distinct regions surrounding ARE-III were found to bind ubiquitous and cell-specific proteins. These regions, when mutated, were shown to be required for maximal activity 68 in the LNCaP prostate cancer cell line.[68] Cell line transfection with a series of 5' deletion constructs of the KLK2 promoter revealed the presence of a functional ARE, although less homologous to the consensus glucocorticoid response element (GRE) and less palindromic than that 69 of KLK3, at position -170,[69] which is the exact position where ARE-I of KLK3 was found. In both KLK2 and KLK3, these response elements were experimentally proven to be activated by androgen. Interestingly, a negative regulatory element was also found in position -468 to -323 of KLK2.[69] Henderson *et al.* identified another ARE at position -3819 to -3805 of the KLK2 promoter which is identical to ARE-II of KLK3.[70] Computer analysis revealed the presence of highly conserved CREB, AP-1 binding site and c-Fos serum response elements at comparable positions in the enhancer regions of both genes.[70] There is also about 75% homology between both promoters in the region between ~-3.5 and 5.2 kb. Apart from KLK2 and KLK3, no obvious TATA boxes were found in the promoter of other kallikreins. Although no typical TATA box or CCAAT sequence was detected in the KLK8 promoter, a weak TATA box-like sequence (TTAAAA) and other transcription factor binding sites were predicted.[46] Two major obstacles exist in predicting the promoter response elements; the inaccurate localization of the transcription start site, and the presence of more than 1 splice variant with more than 1 start codon.

Tissue expression and hormonal regulation of the kallikrein genes

Many kallikreins are transcribed predominantly in few tissues, as indicated by Northern blotting. By using the more sensitive RT-PCR technique, kallikreins were found to be expressed at lower amounts in several other tissues. The tissue expression of all kallikreins, assessed by RT-PCR and or Northern blot, is summarized elsewhere.[63] Interestingly, many kallikreins are expressed in endocrine-related organs. For instance, all kallikreins except KLK8, are expressed in the breast and at least 8 kallikreins are expressed in the ovary and ovarian cell lines. Most of the kallikreins are also expressed, to a variable extent, in the prostate and testis. Given the co-expression of many kallikreins in the same tissue, it is possible that these kallikreins may act in concert in cascade pathways, reminiscent of the coagulation and apoptotic process.

Several reports confirmed that many kallikreins are under steroid hormone regula-

tion in endocrine-related tissues and cell lines.[18, 21, 34, 35, 40, 44, 48, 53, 57, 58, 66, 69, 71] An interesting observation is the tissue-specific pattern of regulation of some genes (*e.g.* the prostate-specific expression of PSA) and the different pattern of hormonal regulation in different tissues; *e.g.* KLK4 is up-regulated by androgen in prostate and breast cancer cell lines [34] and by estrogen in endometrial cancer cell lines.[35] Also, KLK12 was found to be up-regulated by androgens and progestins in prostate cancer cell lines and by estrogens and progestins in breast cancer cell lines.[56] Table I summarizes different reports regarding hormonal regulation of kallikrein genes. A noteworthy pattern of the hormonal regulation is that the centromeric and telomeric groups of kallikreins (KLK1-4 and KLK13-15) are mainly up-regulated by androgens, while the central group is up-regulated mainly by estrogens. It will be interesting to investigate if there is a common control mechanism regulating groups of kallikreins in parallel. Functional characterization of the promoters of all kallikreins will better define the mechanism of kallikrein gene regulation by steroids.

Kallikreins in ovarian cancer

The clinical applicability of kallikreins in cancer is well established. Prostate specific antigen (hK3), and more recently human glandular kallikrein (hK2), are biomarkers for prostate cancer. A more detailed discussion about hK2 and hK3 as cancer biomarkers can be found elsewhere.[72] In addition to hK3 being an established marker for prostate cancer diagnosis and monitoring, recent reports suggest its usefulness as a marker for breast cancer prognosis.[73] With full identification of all members of the kallikrein gene family, accumulating reports suggest that other kallikreins might be also related to hormonal malignancies (for instance, breast, prostate, testicular and ovarian cancers). hK10 (NES1) was cloned by subtractive hybridization from a breast cancer library,[50] and later proposed to act as a tumor suppressor gene.[51] KLK12-14 are all down-regulated in breast cancer [56-58, 74, 75] and KLK15 is up-regulated in prostate cancer.[21] Differential expression of some kallikreins was also reported in testicular cancer.[75-77]

Of particular importance is the relationship between kallikreins and ovarian cancer, which is reported by several research groups. A newly identified kallikrein, KLK6 (zyme/protease M/neurosin), was isolated by differential display from an ovarian cancer library.[41] Underwood *et al.*[47] and Magklara *et al.*[78] have shown that KLK8 (also known as ovasin or TADG-14) is differentially expressed in ovarian cancer. KLK7 was also shown to be up-regulated in ovarian cancer patients.[79] Quantitative analysis of ovarian cancer tissues indicated also that, at the MRNA level, KLK4 and KLK5 are indicators of poor prognosis of ovarian cancer.[80-82] More recently, KLK9 has been shown to be a marker of favourable ovarian cancer prognosis.[49]

At the protein level, hK6 and hK10 were recently reported to be potential serum diagnostic and prognostic markers which are elevated in many ovarian cancer patients.[83, 84] Very recently, hK11 was added to the list of serum ovarian cancer diagnostic biomarkers.[85] Table II summarizes all the available reports describing differential expression of kallikreins at both tissue and serum levels in ovarian cancer.

Is there a steroid hormone-driven enzymatic cascade of kallikreins?

The mechanism by which kallikreins might be involved in the pathogenesis and/or progression of cancer is not yet fully understood. Preliminary reports indicate the possible role of kallikreins in controlling vital processes, like apoptosis, angiogenesis and tumor metastasis by cleavage of specific substrates, including growth factors, hormone receptors or connective tissue. Proteolytic enzymes are thought to be involved in tumor progression because of their role in extracellular matrix degradation. Many studies have shown that a variety of proteolytic enzymes are overproduced either by the cancer cells themselves or by the surrounding stromal cells, and have

TABLE II.—*Clinical applications of the kallikreins and proteins as ovarian cancer biomarkers.*

Gene/ protein sample	Sample used	Method	Application	References
KLK4	mRNA from ovarian cancer tissues	RT-PCR1[1]	Prognosis; overexpression in more aggressive cancers (late stage; higher grade; shorter disease-free and overall survival)	80
	mRNA from ovarian cancer tissues	SQ RT-PCR[2]	Prognosis; overexpression in late stage cancer	82
KLK5	mRNA from ovarian cancer tissues	RT-PCR	Prognosis; overexpression in more aggressive cancers (late stage; higher grade; shorter disease-free and overall survival)	81
KLK6	mRNA from ovarian cancer cell lines and tissues	Northern blot	Higher level of message in some cell lines and tissues. No application specified	41
	Serum	Immunoassay	Diagnosis and monitoring of ovarian cancer	83
	Ovarian cancer cytosols	Immunoasssay	Prognosis; higher levels associated with late stage and decreased-free and overall patient survival	Our unpublished data
KLK7	mRNA from ovarian cancer tissues	SQ RT-PCR	Prognosis; overexpression in ovarian cancer	86
	Ovarian cancer mRNA and extracts	RT-PCR; Northern blots; Western blots; immunohistochemistry	Overexpression of mRNA and protein in the majority of ovarian tumors	79
KLK8	mRNA from ovarian cancer tissue	RT-PCR	Prognosis; higher expression is associated with lower grade and improved patient survival	78
	mRNA from ovarian cancer tissues	Northern blot	Prognosis; high expression in ovarian cancer	47
KLK9	mRNA from ovarian cancer tissues	Q RT-PCR[3]	Prognosis; higher expression is associated with early stages and optimal debulking	49
KLK10	Serum	Immunoassay	Diagnosis and monitoring of ovarian cancer	84
	Ovarian cancer cytosolic extracts	Immunoassy; immunohistochemistry	Prognosis; high levels are associated with late stage disease and decreased disease-free and overall patient survival	87
KLK11	Serum of ovarian cancer	Immunoassay	Diagnosis and prognosis; elevated in 70% of ovarian cancer patient	85
KLK14	Ovarian cancer tissues	Q RT-PCR	Prognosis, higher expression associated with increased survival	Our unpublished data
KLK15	Ovarian cancer tissues	Q RT-PCR	Prognosis, higher expression associated with increase survival	Our unpublished data

1. RT-PCR: reverse transcriptase-polymerase chain reaction; 2. SQ RT-PCR: semiquantitative RT-PCR; 3. Q RT-PCR: quantitative RT-PCR.

shown that their overexpression is associated with unfavorable clinical prognosis.

Ovarian cancer is a "hormonal" malignancy. Sex hormones are known to affect the initiation, and/or progression of ovarian cancer.[88, 89] Oral contraceptive pill administration decreases the risk of ovarian cancer,[1] and the growth of ovarian carcinoma cell lines is sensitive to estrogen.[90] Progesterone promotes cell differentiation and apoptosis, and it has been shown to inhibit DNA synthesis and cell division.[91] Recent studies supported the favorable prognostic value of the progesterone receptor (PR) and its level of expression in ovarian cancer, and indicated that PR-negative status is more abundant in grade 3 ovarian tumors. Appreciable evidence implicates androgens in the pathogenesis of ovarian cancer,[92] and supports the existence of a physiological interaction between androgens and

the ovarian surface epithelium, as well as the possible role of this interaction in ovarian neoplasia.[93] Also, androgens stimulate growth of rodent ovarian epithelial cells *in vivo*, leading to benign ovarian neoplasms.[94] Furthermore, ovarian cancer patients have higher levels of circulating androgens prior to their diagnosis than women without cancer.[95] Additionally, the majority of ovarian cancers express androgen receptor (AR),[96, 97] and ovarian cancer cell growth is inhibited *in vitro* by antiandrogens.[98] Recent observations show a correlation between AR and susceptibility to ovarian cancer.[97, 99-101]

Given the fact that most kallikreins are regulated by sex hormones, we can thus hypothesize that kallikreins are downstream targets by which steroids are involved in the malignant process. The elevation of serum concentration of these biomarkers in cancer is likely due to the increased vasculature (angiogenesis) of cancerous tissues, the destruction of the glandular architecture of the tissues involved and the subsequent leakage of these proteins into the general circulation.

Conclusions

Strong evidence suggests that many kallikrein genes are differentially expressed in ovarian cancer. Some kallikreins are already promising new serum diagnostic/prognostic markers for ovarian cancer. Although the mechanism by which kallikreins are involved in ovarian cancer is yet to be explored, a hormonal theory is supported by preliminary evidence. It will be interesting to study the "global" pattern of kallikrein levels in serum and tissue of ovarian cancer patients compared to normal controls. This multi-parametric model might be very helpful as a diagnostic or prognostic tool.

Riassunto

Esiste un rapporto tra callicreina, ormoni steroidi e carcinoma ovarico?

Le callicreine sono un sottogruppo di proteasi seriche con differenti funzioni fisiologiche. La famiglia dei geni della callicreina umana è stata attualmente ben caratterizzata e risulta comprendere 15 membri localizzati in tandem sul cromosoma 19q13.4. Sono emersi convincenti dati sperimentali a favore dell'esistenza di un legame fra callicreine e neoplasie maligne su base endocrina, in particolare il carcinoma ovarico. Tre callicreine di recente individuazione si sono dimostrate in grado di svolgere il ruolo di potenziali marcatori del carcinoma ovarico, ai fini diagnostici e prognostici. Molte altre callicreine vengono espresse in maniera differenziata dal carcinoma ovarico e osservazioni preliminari ne sottolineano il possibile valore prognostico. Non è noto il meccanismo attraverso il quale le callicreine sono coinvolte nella patologia del carcinoma ovarico. Un verosimile legame potrebbe essere rappresentato dalla partecipazione delle callicreine alla regolazione dei recettori per gli ormoni steroidi. La maggior parte delle callicreine si rivelano regolate dagli steroidi sessuali nelle colture allestite a partire da cellule di carcinoma ovarico. Dal momento che questo esprime contemporaneamente molte callicreine, è ragionevole ipotizzare che le callicreine siano coinvolte in una via enzimatica «a cascata» che svolge un ruolo nella progressione della malattia. Un profilo multiparametrico dell'espressione delle callicreine potrebbe rappresentare un utile mezzo nella valutazione diagnostica e prognostica del carcinoma ovarico, usato da solo oppure in associazione con i marcatori neoplastici attualmente disponibili.

Parole chiave: Callicreine, genetica - Marker tumorali - Ovaio, neoplasie, fisiopatologia - Ormoni steroidei - Prognosi.

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